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Germline and Somatic *Alk* Alterations in Neuroblastoma Patients

Ana Catarina Marinho da Rocha

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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GERMLINE AND SOMATIC *ALK* ALTERATIONS IN NEUROBLASTOMA PATIENTS

Dissertação de candidatura ao grau de Mestre em Oncologia – especialização em Oncologia Molecular – submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

— Marie Curie

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OBRIGADA AVÔ.

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SUMMARY

SUMMARY

Neuroblastoma (NB) is a pediatric cancer of the developing sympathetic nervous system that accounts for more than 7% of all childhood cancers. It is the most common extracranial solid tumor diagnosed during infancy and is responsible for around 10 to 12% of childhood cancer-related deaths. Although most NB tumors arise sporadically, around 1 to 2% of the cases are inherited in an autosomal dominant manner. Anaplastic lymphoma kinase (ALK) is a cell-membrane associated tyrosine kinase receptor, preferentially expressed in the central and peripheral nervous system. Relatively recent findings have suggested that the *ALK* gene may be activated by genomic rearrangements or specific point mutations targeting the tyrosine kinase domain (TKD), thereby contributing to tumor development, and that NB harboring this *ALK* alterations might be sensitive to ALK inhibitors. Additionally, *ALK* germline mutations explain a large proportion of hereditary NB, being present in approximately half of hereditary cases.

In the present study, we aimed to search for *ALK* fusion genes, to compare the pattern of amplification of the *ALK* and *MYCN* genes, to identify somatic and germline *ALK* TKD point mutations and to relate the *ALK* alterations with clinical outcome in NB patients. We therefore performed fluorescence *in situ* hybridization (FISH) in 41 cell suspension tumor samples and Sanger sequencing in 29 fresh-frozen tumor samples from NB patients treated at IPO-Porto from 2003 to 2016 and tested for *MYCN* amplification at the Department of Genetics of that institution. Whenever possible, normal tissue was analyzed from patients presenting mutations in tumor samples.

Of the 41 patients assessed by FISH, no translocation or inversion were found, but aberrant copy number of the *ALK* gene was observed in 19 cases (46.3%). This included *ALK* amplification in one of 41 (2.4%), *ALK* gain in 13 of 41 (31.7%), and *ALK* loss/imbalance in five of 41 (12.2%), changes that in general are associated with more advanced clinical stages and worse outcomes. Additionally, polysomy of chromosome 2 was observed in 15 cases (36.6%), which is an indicator of a better outcome in patients without other adverse prognostic features. Synchronic *MYCN* and *ALK* aberrations accounted for 17 of 41 (41%) tumors. Moreover, we found four different *ALK* TKD mutations in five of 29 tumors tested by Sanger sequencing, namely, the F1174L, R1192P, F1174I, and R1275Q mutations. Of these, the mutations R1192P and R1275Q were found in the germline of two patients, being the most frequent *ALK* germline mutations described in hereditary NB.

We conclude that *ALK* alterations are a frequent event in NB patients, either by cytogenetic events or point mutations, and that these alterations could be a predictive and

prognostic biomarker, which can also be a potential therapeutic target in a subset of patients. We also show the importance of understanding which *ALK* mutations are more likely to be associated with inherited predisposition to NB tumors, which can help direct appropriate screening in families carrying *ALK* germline mutations.

RESUMO

RESUMO

O neuroblastoma (NB) é um cancro pediátrico que surge durante o desenvolvimento do sistema nervoso simpático e que representa mais de 7% de todos os cancros na infância. É o tumor sólido extracraniano mais frequentemente diagnosticado na infância e é responsável por cerca de 10 a 12% da mortalidade por cancro em crianças. Embora a maioria dos NBs sejam esporádicos, cerca de 1 a 2% dos casos são hereditários, com transmissão autossómica dominante. O gene *ALK* codifica um recetor tirosina quinase transmembranar, que é preferencialmente expresso no sistema nervoso central e periférico. Estudos relativamente recentes sugerem que o gene *ALK* pode ser ativado devido à ocorrência de rearranjos cromossómicos ou mutações pontuais no domínio tirosina quinase, contribuindo para o desenvolvimento tumoral. Tumores que apresentam alterações no gene *ALK* podem responder a inibidores do ALK. Além disso, mutações germinativas no gene *ALK* são responsáveis pela maioria dos tumores hereditários, estando presente em aproximadamente metade dos casos.

O presente estudo teve como principais objetivos pesquisar genes de fusão envolvendo o gene *ALK*; comparar o padrão de amplificação dos genes *ALK* e *MYCN*; identificar mutações pontuais somáticas e germinativas no domínio tirosina quinase do gene*;* e, ainda*,* relacionar as alterações encontradas no gene *ALK* com dados clinico-patológicos dos pacientes com NB. Foi utilizada a técnica de FISH em 41 amostras de células tumorais em suspensão e sequenciação de Sanger em 29 amostras de tumores frescos congelados de pacientes com NB tratados no IPO-Porto de 2003 a 2016 e testados para amplificação do gene *MYCN* no Serviço de Genética daquela instituição. Sempre que possível, foi efetuada a análise de tecido normal dos pacientes com mutação no gene *ALK* na amostra tumoral.

Dos 41 pacientes avaliados por FISH, não foram encontrados casos com translocação ou inversão, mas foi observado um número de cópias aberrante do gene *ALK* em 19 casos (46,3%). Destes, foi identificada amplificação em um caso (2,4%), ganho do *ALK* em 13 casos (31,7%) e perda/desequilíbrio do *ALK* em cinco casos (12,2%), alterações que em geral estão associadas a estádios clínicos mais avançados e a pior prognóstico. Adicionalmente, foi observada polissomia do cromossomo 2 em 15 casos (36,6%), que está associada a um melhor prognóstico em pacientes sem outras características de mau prognóstico. A co-ocorrência de alterações nos genes *MYCN* e *ALK* foi detetada em 17 casos (41%). Além disso, encontrámos quatro mutações no domínio tirosina quinase do gene *ALK* em cinco dos 29 tumores analisados por sequenciação de Sanger, nomeadamente, as mutações F1174L, R1192P, F1174I e R1275Q. Destas, as

mutações R1192P e R1275Q foram encontradas na linha germinativa de dois pacientes, sendo as mais frequentemente reportadas no NB hereditário.

Concluímos assim que alterações no gene *ALK* são um evento frequente em pacientes com NB, quer seja devido a eventos citogenéticos ou à ocorrência de mutações pontuais. Estas alterações poderão ser utilizadas como biomarcador preditivo e de prognóstico, podendo também ser um potencial alvo terapêutico. Mostrámos também a importância de entender que mutações no gene *ALK* estão associadas com predisposição hereditária, permitindo orientar o rastreio nas famílias com mutações germinativas no gene *ALK.*

LIST OF ABREVIATIONS

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aCGH – array-based comparative genomic hybridization

AHSCT – autologous hematopoietic stem-cell transplantation

ALCL – anaplastic large-cell lymphoma

BM – basement membrane

CCHS – congenital central hypoventilation syndrome

CNS – central nervous system

DAPI – 4', 6-diamidino-2-phenylindole

ddNTP – Dideoxynucleotide

DLBCL – diffuse large B cell lymphoma

DNA – Deoxyribonucleic acid

dNTP – deoxynucleoside triphosphate

ECM – extracellular matrix

EFPE – formalin-fixed paraffin-embedded

EFS – event-free survival

ERK – extracellular signal-regulated kinase

FISH – Fluorescence *in situ* hybridization

GM-CSF – granulocyte-macrophage - colony-stimulating stimulating factor

GN – Ganglioneuroma

GNB – Ganglioneuroblastoma

GWAS – genome-wide association studies

HSCR – Hirschsprung disease

ICCC – International Classification of Childhood *Cancer*

IHC – immunohistochemistry

IL2 – interleukin 2

IMT – inflammatory myofibroblastic tumor

INRG – International Neuroblastoma Risk Group

INRGSS – International Neuroblastoma risk group staging system

INSS – International Neuroblastoma Staging System

JAK – Janus kinase

LOH – loss of heterozygosity

MAPK – mitogen activated protein kinase

MK – midkin

MKI – Mitosis Karyorrheris Index

MLPA – multiplex ligation-dependent probe amplification

mRNA – messenger ribonucleic acid

NB – Neuroblastoma

NSCLC – non-small-cell lung cancer

PCR – polymerase chain reaction

PI3K – phosphoinositide 3-kinase

PTN – pleiotropin

RAS – rat sarcoma oncogene

RCC – renal cell carcinoma

RT – room temperature

SCC – squamous cell carcinoma

SNP – single nucleotide polymorphism

STAT – signal transducer and activator of transcription

TKD – tyrosine kinase domain

GENES

***ALK* –** anaplastic lymphoma kinase

***APC* –** adenomatous polyposis coli

***ARID1A* –** AT-rich interaction domain 1A

***ARID1B* –** AT-rich interaction domain 1B

***ATRX* –** ATP-dependent helicase

***BARD1* –** BRCA1-associated ring domain 1

***CASC15* –** cancer susceptibility candidate 15

***CHEK2* –** checkpoint kinase 2

***CLPTM1L* –** cleft lip and palate transmembrane protein 1-like protein

***DDX4* –** DEAD (Asp-Glu-Ala-Asp) box polypeptide 4

***DUSP12* –** dual specificity phosphatase 12

***EML4* –** echinoderm microtubule-associated protein-like 4

GAB2 – GRB2 Associated Binding Protein 2

***HACE1* –** repeat containing E3 ubiquitin protein ligase 1

***HRAS* –** Harvey rat sarcoma viral oncogene homolog

***IL31RA* –** Interleukin 31 receptor A

***LMO1* –** LIM domain only 1

***MYCN* –** MYCN proto-oncogene, bHLH transcription factor

***NF1* –** neurofibromin 1

***NPM* –** nucleophosmin

***PHOX2B* –** paired-like homeobox 2B

***PINK1* –** PTEN-induced putative kinase 1

***PTPN11* –** protein tyrosine phosphatase, nonreceptor type 11

***SDHB* –** succinate dehydrogenase complex iron sulfer, subunit B

***TERT* –** telomerase reverse transcriptase

***TIAM1* –** T-cell lymphoma invasion and metastasis 1

***TP53* –** tumor protein 53

I. INTRODUCTION

I. INTRODUCTION

Cancer arises from the progressive accumulation of genetic and epigenetic alterations on cells, that acquire the ability to uncontrollable proliferation, suppression of apoptosis, invasion, metastization and stimulation of angiogenesis (Tornesello et al., 2015).

Cancer is a major health problem and is one of the most common cause of death worldwide (Ferlay et al., 2015; Torre et al., 2015). In general, cancer rates are higher in more developed countries, however, these have been increasing in developing ones, maybe due to the adoption of lifestyle behaviors that increase cancer risk, such as smoking, a poor diet, sedentariness and reproductive changes (Torre et al., 2015). Endogenous factors may also influence the individual risk for cancer, such as endogenous hormones, medical history and/or genetic susceptibility (Siegel et al., 2016).

Although cancer incidence increases with age, there is a small proportion of cases, less than 2% of the global cancer burden, diagnosed in children and young adults (aged 0 to 19 years) (Pritchard-Jones and Sullivan, 2013). In more developed countries, cancer is the second commonest cause of childhood mortality (Kaatsch, 2010; Siegel et al., 2016).

Childhood cancer is classified according to the International Classification of Childhood Cancer (ICCC). On the first year of life, neuroblastomas, retinoblastomas and nephroblastomas combined account for about half of all malignancies, while leukemias, central nervous system (CNS) tumors and lymphomas predominate in ages ranging from 1 to 14 years. In adolescents (aged 15 to 19 years), a smaller proportion of cancers diagnosed are leukemias and a larger proportion are lymphomas and CNS tumors (Kaatsch, 2010; Siegel et al., 2016). Among all ages (0 to 19 years), brain cancer is the commonest leading cause of cancer death (Siegel et al., 2016).

Cancer incidence rates increased in children and adolescents by 0.6% per year from 1975 to 2012. In contrast, the 5-year relative survival rate improved from 58% to 83%, for all cancer sites combined (Siegel et al., 2016). The improvements in treatment and the novel approaches to the design of clinical trials may explain the substantial progress for all major childhood cancers.

1. Neuroblastoma

The term neuroblastoma (NB) is commonly used to refer to a spectrum of peripheral neuroblastic tumors, including neuroblastoma, ganglioneuroblastoma (GNB) and ganglioneuroma (GN). NB is a malignancy of early childhood that arises from the aberrant growth of neural crest progenitor cells during the development of sympathetic nervous system (Maris, 2010).

1.1. Epidemiology

NB is the most frequent extracranial solid tumor in childhood accounting for more than 7% of malignancies in patients younger than 15 years-old. This pediatric cancer varies greatly between age groups, with a peak of incidence in children less than four years old, with boys being, in general, more affected than girls (Kaatsch, 2010; Park et al., 2013).

The median age at diagnosis of NB patients is about 18 months (Matthay et al., 2016), and often presents with widespread metastatic disease, resulting in survival rates less than 50% (Maris, 2010; Pugh et al., 2013). It accounts for about 10 to 12% of childhood cancer mortality (Park et al., 2013; Pugh et al., 2013).

The age-standardized incidence rate varies internationally. A relatively higher incidence is observed in developed countries than in developing ones. The incidence rate of NB in Europe, about 11.2 per million children (Kaatsch, 2010), is very close to that in North America, which is about 10.5 per million children (Park et al., 2013). Furthermore, differences between Black and White races are observed. Black race from both Africa and North America seems to show a significantly lower NB incidence rate than Caucasians (Stiller and Parkin, 1992). However, in Black race, NB is diagnosed at an older age, with a higher prevalence of high-risk disease and a worse outcome (Henderson et al., 2011; Stiller and Parkin, 1992).

In Portugal, the incidence rate of NB is not established. The crude incidence of malignant childhood cancers diagnosed in Portugal from 2000 to 2006 was 9.2 per 100 000 per year, comparable to 14.8 in Southern Europe, and an improvement of 5-year age-standardized survival has been recorded from 1999 to 2007 (Gatta et al., 2014). Additionally, the data of children diagnosed with NB in Southern Europe showed an improvement of survival, from 65% to 71.9% (Gatta et al., 2014).

1.2. Diagnostic and risk classification

Determination of tumor staging and its biology is performed at the time of diagnosis and requires a range of exams that include analysis of different plasmatic and urinary parameters, radiographic images and histological and genetical assessment. Therapeutic decision is based on stratification into different risk groups according to the International Neuroblastoma Risk Group (INRG).

The normal neural crest cells differentiate into sympathetic neurons, chromaffin, Schwann and satellite cells, however, NB tumors contain immature sympathetic neurons and Schwann cells (Ratner et al., 2016). In 1984, Shimada et al. introduced a new terminology and classification of peripheral neuroblastic tumors. Tumors were classified as favorable or unfavorable histology according to the amount of Schwannian stroma (stroma-poor or stroma-rich), degree of neuroblast differentiation, patient's age at diagnosis and Mitosis Karyorrheris Index (MKI) of the tumor.

NB tumors are assigned to one of four morphologic categories based on the scheme proposed by Shimada et al. (1984) and morphologic features described by Joshi and colleagues (Joshi et al., 1992; Joshi et al., 1996): neuroblastoma (Schwannian stroma-poor); ganglioneuroblastoma, intermixed (Schwannian stroma-rich); ganglioneuroma (Schwannian stroma-dominant), and ganglioneuroblastoma, nodular (composite Schwannian stroma-rich/stroma-dominant and stroma-poor) (Shimada et al., 1999) (Supplementary Table 1).

The International Neuroblastoma Staging System (INSS) was stablished in 1986, for a common diagnosis and staging (Supplementary Table 2). (Brodeur et al., 1993). However, a new staging scheme, the International Neuroblastoma Risk Group staging system (INRGSS), was proposed in 2009 by Monclair et. al, based on clinical criteria and tumor imaging rather than extent of surgical resection, allowing a pretreatment staging and risk assessment (Table I.1).

Table I.1: International Neuroblastoma Risk Group Staging System (Monclair et al., 2009).

Stage	Definition
L1	Localized tumor not involving vital structures as defined by the list of imaging-defined risk factors and confined to one body compartment.
L2	Locoregional tumor with presence of one or more image-defined risk factors.
M	Distant metastatic disease (except stage MS).
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow.

After disease staging, each patient is stratified, based on clinical and molecular risk factors (such as *MYCN* amplification, 11q aberration and ploidy) as very low-risk, low-risk, intermediate-risk or high-risk, with 5-year event-free survival (EFS) of more than 85%, 75% to ≤ 85%, ≥ 50% to ≤ 75%, or less than 50%, respectively (Cohn et al., 2009). The stratification in pretreatment risk groups, summarized in Table I.2, helps the clinicians to decide the best course of treatment (Pinto et al., 2015).

Table I.2: International Neuroblastoma Risk Group pretreatment classification schema (Pinto et al., 2015).

INRG Stage	Age (months)	Histologic category	Grade of tumor differentiation	MYCN	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing, GNB intermixed					A (very low)
L1		Any, except GN maturing or GNB intermixed		NA			B (very low)
				Amplified			K (high)
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D (low)
					Yes		G (intermediate)
	≥ 18	GNB nodular neuroblastoma	Differentiating	NA	No		E (low)
					Yes		H (intermediate)
			Poorly differentiated or undifferentiated	NA			H (intermediate)
				Amplified			N (high)
M	< 18			NA		Hyperdiploid	F (low)
	< 12			NA		Diploid	I (intermediate)
	12 to 18			NA		Diploid	J (Intermediate)
	< 18			Amplified			O (high)
	≥ 18						P (high)
MS	< 18			NA	No		C (very low)
					Yes		Q (high)
				Amplified			R (high)

GN: Ganglioneuroma; GNB: Ganglioneuroblastoma; NA: non-amplified

1.3. Clinical presentation

Primary tumors can arise anywhere in the sympathetic nervous system. The majority of tumors have an abdominal location, with at least half of these arising in the adrenal medulla. The neck, chest and pelvis are other sites of disease occurrence (Maris et al., 2007). The location of primary tumor is associated with different disease clinical and biological features. Abdominal and extra-abdominal sites are associated with lower and higher survival, respectively, and tumors located in thorax are more predominant in younger age patients (Vo et al., 2014).

Metastatic disease is detected in approximately half of patients at diagnosis, being the most common locations of metastasis the regional lymph nodes, cortical bone and bone marrow (Maris et al., 2007; Matthay et al., 2016). On the other hand, metastatic involvement of lung and brain is rare (DuBois et al., 1999). However, there is a group of patients with metastization to the liver, skin and bone marrow in children with less than 18 months of age, which represents a clinical stage designed MS (D'Angio et al., 1971; Evans et al., 1971). This specific stage is diagnosed in about 5% of the cases and almost always regress spontaneously (DuBois et al., 1999; Maris et al., 2007; Matthay et al., 2016).

The presentation of symptoms depends on the site of the primary tumor, the extension of metastatic disease and the presence or absence of paraneoplastic syndromes (Maris et al., 2007; Matthay et al., 2016).

1.4. Treatment

NB treatment includes surgery, chemotherapy, radiotherapy, high-dose chemotherapy/radiotherapy with autologous stem cell transplant. It can also be used retinoid acid therapy, immunotherapy, and more recently targeted therapy (Pinto et al., 2015; Simon et al., 2017).

Low- or intermediate-risk patients have excellent outcomes. For low-risk patients without symptoms, surgery alone can be curative. Symptomatic low-risk patients at diagnosis can receive a less-intensive chemotherapy without radiotherapy. However, some observational studies demonstrated a subset of infants with localized tumors that can be cured without any treatment, including surgery (Pinto et al., 2015; Simon et al., 2017). Regarding the intermediate-risk patients, a more intensive regimen of chemotherapy and radiotherapy is needed for older patients with unfavorable histology tumors (Pinto et al., 2015).

Treatment regimens of high-risk patients include induction chemotherapy and surgery, consolidation chemotherapy with myeloablative chemotherapy with autologous hematopoietic stem-cell transplantation (AHSCT) and irradiation, and post consolidation therapy to treat minimal residual disease (Pinto et al., 2015) (Figure I.2).

Resection of the primary tumor is performed during or after induction therapy and the disadvantage of incomplete resection might be compensated by intensified local radiotherapy (Simon et al., 2017). Induction therapy consists in a combination of agents, including cisplatin, cyclophosphamide, carboplatin, doxorubicin, etoposide, topotecan and vincristine (Pinto et al., 2015; Simon et al., 2017). Consolidation therapy consists in myeloablative chemotherapy regimens with autologous stem cell transplantation. External beam radiotherapy is recommended followed by myeloablative chemotherapy and AHSCT

for cases with residual tumor tissue present after surgery and induction therapy (Simon et al., 2017).

Despite the improvement in EFS with myeloablative chemotherapy followed by AHSCT, all patients who achieve a clinical remission relapse months to years after transplantation. A post-consolidation therapy with the combination of the ch14.18/SP2/0 anti-GD2 antibody (Dinutuximab), CSF2 (formerly granulocyte-macrophage colony-stimulating factor or GM-CSF), interleukin 2 (IL2) and isotretinoin is considered a standard first-line treatment for these patients (Matthay et al., 2016; Pinto et al., 2015; Simon et al., 2017).

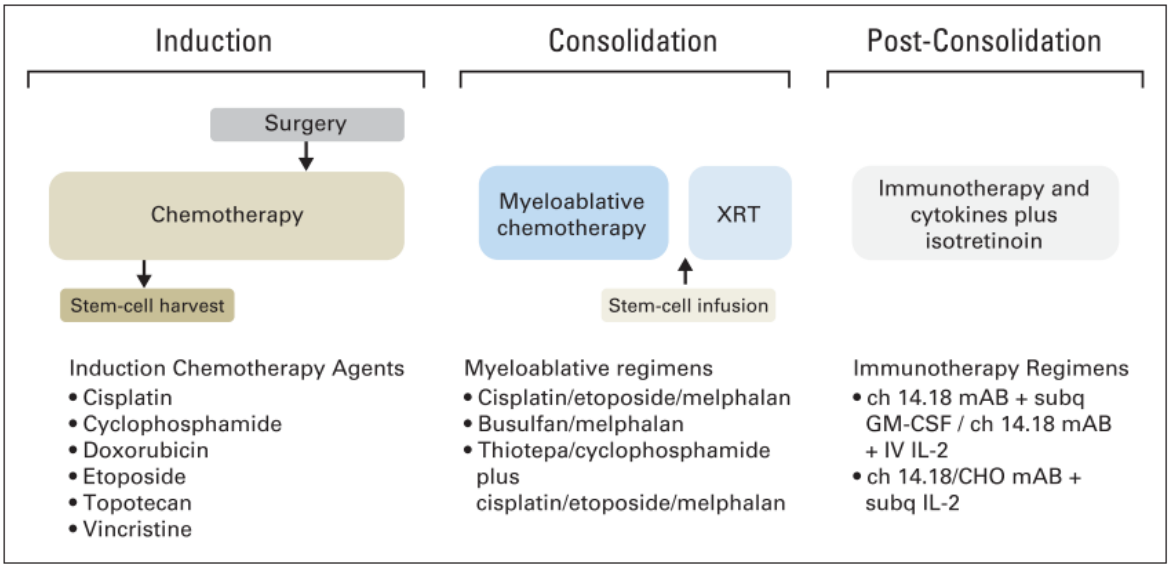

Figure I.1: Current standard-of-care treatment strategy for high-risk neuroblastoma (NB) (Pinto et al., 2015)**.**

2. Genetic alterations in NB

2.1. Germline mutations

An hallmark of NB is its heterogeneity in clinical presentation, course and overall prognosis (Bosse and Maris, 2016). It presents several types of unique biologic behaviors, such as spontaneous regression, maturation and high proliferation (Shimada et al., 1999), but, its etiology is largely unknown.

About 1 to 2% of NB are inherited, in an autosomal dominant manner (Azarova et al., 2011; Mosse et al., 2008). Germline mutations in the anaplastic lymphoma kinase (*ALK*) and paired-like homeobox 2B (*PHOX2B*) genes are responsible for the majority of hereditary cases (Azarova et al., 2011; Parodi et al., 2014). Activating mutations in the *ALK* gene, located on chromosome 2p23, are the germline alterations more frequently

associated with hereditary NB, being identified in about 50% of the cases (Mosse et al., 2008; Schleiermacher et al., 2014).

There is an association of NB with other genetically determined neurocristopathies, such as Hirschsprung disease (HSCR) and/or congenital central hypoventilation syndrome (CCHS). Alterations in *PHOX2B* gene, located on chromosome 4p, are the major disease-causing of CCHS, but, germline mutations have also been identified in NB patients with HSCR and/or CCHS (Mosse et al., 2004; Trochet et al., 2004). However, *PHOX2B* mutations explain only a small proportion of hereditary NB (less than 10%) (Mosse et al., 2008; Raabe et al., 2008). Although more rarely, few NB cases are associated with genetic syndromes with underlying rat sarcoma oncogene-mitogen activated protein kinase (RAS-MAPK) pathway germline mutations, such as Neurofibromatosis type 1, Noonan syndrome and Costello syndrome (Figure I.1 [left]) (Bosse and Maris, 2016; Schleiermacher et al., 2014).

Mutations in other genes that can predispose to NB have been identified, but the clinical relevance of these alterations remains poorly understood. In 2013, Pugh *et al.* found the tumor protein 53 (*TP53*) p.Pro219Ser mutation in NB patients, consistent with prior reports of NB being weakly associated with Li-Fraumeni syndrome (Birch et al., 2001). Two other germline variants in the *TP53* gene were found as being strongly associated with NB (Diskin et al., 2014). Other studies have identified putative damaging mutations in checkpoint kinase 2 (*CHEK2*), PTEN-induced putative kinase 1 (*PINK1*), BRCA1-associated ring domain 1 (*BARD1*), adenomatous polyposis coli (*APC*) and succinate dehydrogenase complex iron sulfur, subunit B (*SDHB*) genes in small percentages of NB patients (Figure I.1 [middle]) (Pugh et al., 2013; Zhang et al., 2015).

Genome-wide association studies (GWAS) identified common DNA variations that can influence the malignant transformation in NB. Common single nucleotide polymorphisms (SNPs) were identified in cancer susceptibility candidate 15 (*CASC15*), *BARD1*, LIM domain only 1 (*LMO1*), HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (*HACE1*), which were associated with high-risk of NB development (Capasso et al., 2009; Diskin et al., 2012; Russell et al., 2015; Wang et al., 2011). On the other hand, the SNPs identified in dual specificity phosphatase 12 (*DUSP12*), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*DDX4*) and interleukin 31 receptor A (*IL31RA*) were associated with the low-risk NB (Figure I.1 [right]) (Bosse and Maris, 2016; Nguyen le et al., 2011).

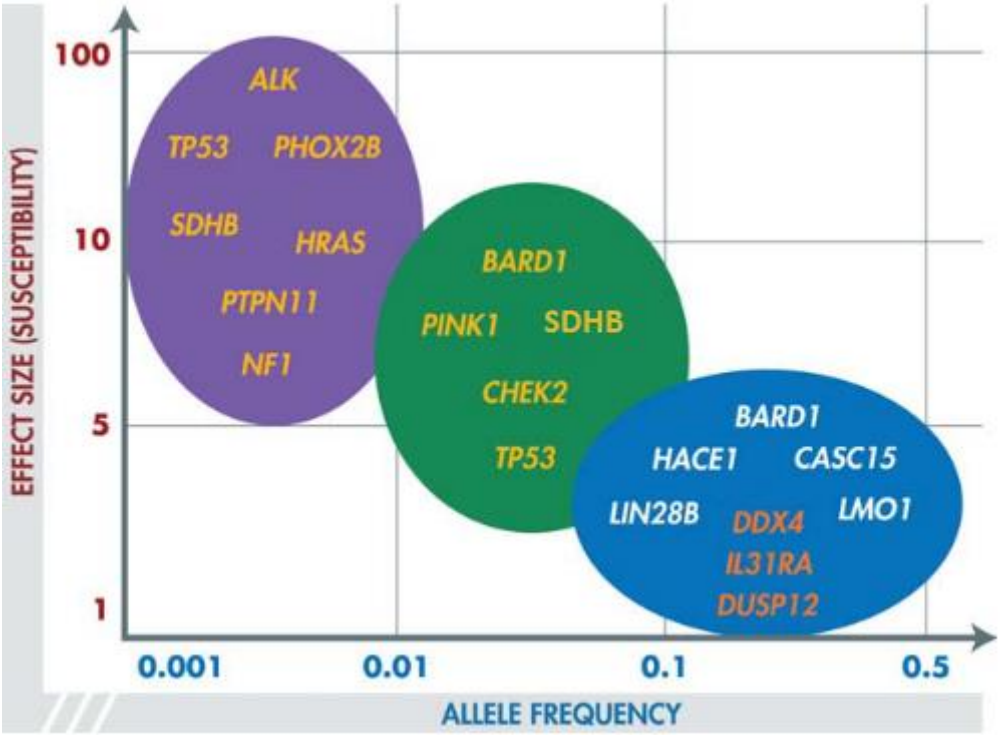

Figure I.2: Rare and common genomic variants that predispose to NB. [left] mutations in anaplastic lymphoma kinase (*ALK*) and paired-like, homeobox 2B (*PHOX2B*) are inherited in an autosomal dominant Mendelian manner, with incomplete penetrance. Additionally, NB can also arise in a subset of genetic syndromes with germline mutations in tumor protein 53 (*TP53*), succinate dehydrogenase complex iron sulfur, subunit B (*SDHB*), Harvey rat sarcoma viral oncogene homolog (*HRAS*), protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*) and neurofibromin 1 (*NF1*). [middle] Low-frequency damaging mutations with an intermediate effect size. [right] More common alleles with a modest effect size, specifically to high-risk (white) or low-risk (orange) NB [Adapted from (Bosse and Maris, 2016)].

2.2. Chromosomal changes

Oncogenes appear to be activated from their normal state due to mutations, gene amplification or gene fusion (Ponder, 1992). In 1984, the amplification of *MYCN* gene, located on chromosome 2p24.1, was described for the first time in NB patients (Brodeur et al., 1984). *MYCN* amplification, present in approximately 20 to 25% of all primary tumors (Maris et al., 2007), is associated with advanced stages of disease and with poor prognosis (Brodeur et al., 1984; Seeger et al., 1985). Around 6% of tumors show only one or a few additional *MYCN* copies, defined as *MYCN*-gain, which is also associated with a worse prognosis in diploid tumors (Spitz et al., 2004). Additionally, deletion of chromosome 11q, which is present in 35 to 45% of the cases, is inversely associated with *MYCN* amplification (Maris et al., 2007), however, it is correlated with *MYCN* copy number gain (Spitz et al.,

2004). Therefore, the presence of deletion of 11q has emerged as a powerful prognosis biomarker of a worse prognosis in patients without *MYCN* amplification (Attiyeh et al., 2005).

Additionally, other chromosome changes have been identified with prognostic value in NB patients, including deletions of chromosomes 1p, 3p, and 4p, and a gain of 1 to 3 copies of chromosome 17q region (Janoueix-Lerosey et al., 2009). In general, tumors with gains of whole-chromosome, resulting in hyperdiploidy, are associated with favorable features such as non-metastatic disease or stage MS and younger age at diagnosis, whereas tumors with segmental chromosome alterations are associated with more aggressive forms of disease. The tumor overall genomic alteration pattern defines specific genetic groups with different clinical outcomes (Bosse and Maris, 2016; Janoueix-Lerosey et al., 2009; Oberthuer et al., 2009).

These segmental genetic aberrations can be assessed by interphase fluorescence *in situ* hybridization (FISH), array-based comparative genomic hybridization (aCGH), polymerase chain reaction (PCR) or multiplex ligation-dependent probe amplification (MLPA) techniques (Ambros et al., 2009). The assessment of tumor cell DNA content (ploidy) was shown to influence the prognosis in children younger than 2 years at diagnosis (Look et al., 1991; Maris et al., 2007) and is assessed by flow or static cytometry, being classified as diploid or hyperdiploid (near-triploid or penta/hexaploidy) (Ambros et al., 2009).

Allelic loss of chromosome 1p, identified in 25 to 35% of all NB, seems to be independent of patient's age and stage of disease, and is one of the most powerful prognostic indicators of a worse outcome (Maris et al., 2007). A strong correlation exists between *MYCN* amplification and chromosome 1p loss of heterozygosity (LOH), is demonstrated, both correlated with poor clinical outcome and occurring together mainly in more advanced stages and in patients older than one-year (Caron et al., 1996; Fong et al., 1989; Spitz et al., 2002). Aberrations of chromosome 17q are the most common chromosomal alterations described in NB patients, occurring in at least 50% of the tumors (Bosse and Maris, 2016; Maris et al., 2007). The chromosome 17q gain is characteristic of advanced tumors and are found in patients older than one-year, being strongly associated with deletion of 1p and *MYCN* amplification. The principal mechanism involved in partial gain of 17q is an imbalanced translocation with 1p (Bown et al., 1999; Caron et al., 1996).

2.3. Somatic point mutations

Although chromosomal aberrations are common in NB, with a well-stablished influence in patient's outcome, point mutations are quite rare. Some studies of whole-genome sequencing have described mutations in cancer-related genes in NB tumors, which occur more frequently in high-risk patients. Altogether, these studies showed the

occurrence of *ALK* activating mutations in 7 to 14% of sporadic cases, *ALK* amplification in approximately 2% and more rarely other cytogenetic rearrangements of this gene (Molenaar et al., 2012; Mosse et al., 2008; Pugh et al., 2013). Mutations and deletions in ATP-dependent helicase (*ATRX*) gene have been associated with children older than 5 years old with metastatic disease and have been described as mutually exclusive with *MYCN* amplification. *ATRX* plays a significant role in regulating adenosine triphosphate – dependent chromatin remodeling, nucleosome assembly, and telomere maintenance. These mutations were associated with a lower *ATRX* mRNA expression and long telomeres (Cheung et al., 2012; Molenaar et al., 2012; Pugh et al., 2013).

Rearrangements in the telomerase reverse transcriptase (*TERT*) gene were also found in 23% of high-risk NB. Increased *TERT* gene copy number appears to promote metastasis, in absence of *MYCN* amplification (Cobrinik et al., 2013; Valentijn et al., 2015). Chromothripsis, a located shredding of a chromosomal region with subsequent random reassembly of the fragments, has been found in 18% of high-risk patients, and was also associated with a poor prognosis (Molenaar et al., 2012). Additionally, an intragenic hemizygous deletion targeting the AT-rich interaction domain 1B (*ARID1B*) gene and an insertion mutation in the homologous AT-rich interaction domain 1A (*ARID1A*) gene, both chromatin remodeling genes, were found in 11% of NB cases and were associated with early treatment failure and lower survival (Sausen et al., 2013). Although more rarely, mutations in T-cell lymphoma invasion and metastasis 1 (*TIAM1*) gene (3%), a central regulator of cellular polarity and neuritogenesis (Molenaar et al., 2012), and in *PTPN11* gene (2.9%) have also been described (Pugh et al., 2013).

3. The anaplastic lymphoma kinase (*ALK*) gene

The *ALK* gene, located on chromosome 2p23, codes for a membrane associated tyrosine kinase receptor, which is a member of the insulin receptor protein-tyrosine kinase superfamily. The *ALK* gene encodes a mature protein of 220 kDa that migrates as two protein isoforms: the 220 kDa full-length receptor and the truncated 140kDa protein that results from extracellular cleavage. This protein has an extracellular domain, a transmembrane segment and an intracellular domain, which contains a protein kinase domain (Azarova et al., 2011; Roskoski Jr, 2013). It is thought that, in mammalians, *ALK* plays a key role in the development and function of nervous system during the neural development. This gene is preferentially expressed in brain and spinal cord, being highly expressed in neonatal brain (Iwahara et al., 1997; Roskoski Jr, 2013).

The normal *ALK* activation by the growth factors ligands pleiotropin (PTN) and midkine (MK) results in the activation of many different pathways that are strictly

interconnected and overlapping. These include the rat sarcoma oncogene-extracellular signal-regulated kinase (RAS-ERK) pathway, the janus kinase 3-signal transducer and activator of transcription 3 (JAK3-STAT3) pathway and the phosphoinositide 3-kinase-Akt (PI3K-Akt) pathway, which participate in cell proliferation, cell survival, inhibition of apoptosis and also in induction of neuronal cell differentiation through the MAPK pathway (Azarova et al., 2011). A variety of *ALK* gene alterations have been described in several types of tumors, including mutations, amplifications, deletions and translocations leading to an aberrant activity of *ALK* (Chiarle et al., 2008).

3.1. *ALK* changes in various cancers

ALK seems to be a "hotspot" for translocation to a wide variety of loci. A translocation involving chromosomes 2p and 5q, t(2;5)(p23;q35), which generates a fusion protein (NPM-ALK), was described for the first time in the anaplastic large-cell lymphoma (ALCL), the first disorder associated with ALK fusion proteins (Morris et al., 1994; Roskoski Jr, 2013). Since then, 22 other genes have been described as rearranged with *ALK* in several types of cancer, including inflammatory myofibroblastic tumor (IMT), non-small-cell lung cancer (NSCLC), diffuse large B cell lymphoma (DLBCL), squamous cell carcinoma (SCC) and renal cell carcinoma (RCC) (Hallberg and Palmer, 2013). Furthermore, NSCLC patients harboring rearrangements involving *ALK* (*ALK-EML4,* most frequently) are eligible for targeted therapy with ALK inhibitors (Solomon et al., 2014).

More recently, increased *ALK* activity due to overexpression of *ALK* or point mutations has been reported as playing a primary role in the pathogenesis, aggressiveness and lethality of NB (Roskoski Jr, 2013).

3.2. *ALK* rearrangements in NB

Since 2002, several works were published reporting the high incidence of *ALK* mutations and gene amplifications in advanced sporadic NB tumors (Bagci et al., 2012; Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Miyake et al., 2002; Mosse et al., 2008; Osajima-Hakomori et al., 2005; Subramaniam et al., 2009).

Miyake et al. (2002) and Osajima-Hakomori et al. (2005) showed that the constitutively activation of *ALK* gene by amplification plays a role in the pathophysiology of NB, resulting in hyperphosphorylation of ShcC, MAPK and Akt proteins. They showed that *ALK* inhibition suppresses tyrosine phosphorylation of ShcC and blocks MAPK and Akt, resulting in apoptosis. Furthermore, *ALK* amplification has been identified in several studies in approximately 2% of cases and is associated with a worse outcome (Azarova et al.,

2011). Additionally, *ALK* has been shown to be co-amplified with *MYCN* gene, which is located on 2p24.3 very close to the *ALK* locus, in around 7 to 15% of all *MYCN* amplified tumors (Azarova et al., 2011; Bagci et al., 2012; De Brouwer et al., 2010; Wang et al., 2013). However, Chen et al. (2008) suggested that *ALK* and *MYCN* loci can also be amplified in separate amplicons.

Additionally, there is a high frequency of tumors harboring a partial gain of chromosome 2p region, encompassing the *ALK* gene (17 to 45%) (Bagci et al., 2012; Carén et al., 2008; Chen et al., 2008; Mosse et al., 2008). Moreover, *ALK* gene copy number is strongly correlated with mRNA and protein expression levels, which is significantly correlated with a worse overall survival (De Brouwer et al., 2010; Passoni et al., 2009).

More recently, a new mechanism of aberrant *ALK* activation by balanced or unbalanced translocations and deletions has been suggested (Table I.3). A balanced translocation between the *ALK* gene in chromosome 2 and the *CLPTM1L* gene in chromosome 5 was described in a cell line, resulting in an out-of frame fusion transcript, but its biologic function remains to be defined (Cazes et al., 2013). Unbalanced translocations between chromosome 2 and chromosomes 4 and 11, resulting in 2p gain, were described in tumors samples, nevertheless, since gene fusion occurs in opposing transcriptional directions, a chimeric transcript it is unlikely to be produced (Fransson et al., 2015). Additionally, three different deletions, combined with *ALK* locus amplification, were described as leading to truncated ALK variants, which exhibit oncogene properties and are associated with tumor aggressiveness (Cazes et al., 2013; Fransson et al., 2015; Okubo et al., 2012). This pattern of rearrangements and the multiple breakpoints observed in NB tumor samples may correspond to the phenomenon of chromothripsis, recently reported in aggressive NB (Cazes et al., 2013; Fransson et al., 2015; Molenaar et al., 2012; Wang et al., 2013).

Table I.3: *ALK* gene rearrangements described in NB.

Type of rearrangement	Sample	Chromosomes	Genes		References
Balanced translocation	Cell line	2, 5	*ALK*, *CLPTM1L*		(Cazes et al., 2013)
Interchromosomal	Tumor	2, 11	*ALK*, *GAB2*		(Fransson et al., 2015)
Interchromosomal	Tumor	2, 4	*ALK*, subtelomeric region		(Fransson et al., 2015)
Deletion	Tumor	2	*ALK*	Loss exons 1 to 4	(Fransson et al., 2015)
Deletion	Cell line/ Tumor	2	*ALK*	Loss exons 4 to 11	(Cazes et al., 2013)
Deletion	Cell line	2	*ALK*	Loss exons 2 to 3	(Okubo et al., 2012)

3.3. Somatic *ALK* point mutations in NB

Somatic mutations have been identified in approximately 7 to 14% of NB patients, mainly occurring in the catalytic loop or the C-helix kinase domains (Table I.4) (Mosse et al., 2008). Two mutational hotspots, at positions R1275 on exon 25 and F1174 on exon 23, have been described. These mutations occur in the kinase domain and result in the constitutive active forms of the protein, leading to constitutive phosphorylation of ALK and, consequently, phosphorylation of downstream targets, such as STAT3, Akt and ERK (Chen et al., 2008; De Brouwer et al., 2010; George et al., 2008). These mutations were found across the entire spectrum of disease, presenting similar frequencies in cases with or without *MYCN* amplification (De Brouwer et al., 2010; Mosse et al., 2008). Furthermore, no differences in survival of patients with *ALK* mutations were observed, suggesting that *ALK* mutations themselves are not an adverse prognostic factor (Chen et al., 2008; De Brouwer et al., 2010). However, when the survival was compared between different mutations, patients with a F1174 mutation have shown a worse survival than those with a R1275 mutation or wild-type, which can be explained by the high frequency of *MYCN* amplification in F1174 mutation carriers (De Brouwer et al., 2010). Moreover, *ALK* copy number gain was not frequently found in tumors harboring *ALK* mutations, suggesting that 2p gain is not a common mechanism of increasing mutated *ALK* expression (De Brouwer et al., 2010; Passoni et al., 2009).

The clinical role of *ALK* mutations or amplification remains controversial (Bagci et al., 2012; Brodeur et al., 1984; Carén et al., 2008; Chen et al., 2008; De Brouwer et al., 2010). However, it seems to be clear that more aggressive and metastatic tumors exhibit a higher expression of ALK comparatively to localized NB, even without *ALK* gene alterations, suggesting that increased *ALK* expression might be functionally relevant in NB. It is possible that patients with wild-type ALK overexpression might benefit from ALK inhibitors therapy, in addition to those harboring *ALK* amplification or point mutations (Hallberg and Palmer, 2013; Passoni et al., 2009).

Table I.4: *ALK* somatic mutations described in NB patients.

Exon	DNA	Protein		References
20	c.3185A>T	K1062M	Cell line	(Chen et al., 2008; Okubo et al., 2012)
20	c.3271G>A	D1091N	Tumor and cell lines	(De Brouwer et al., 2010; George et al., 2008; Mosse et al., 2008)
22	c.3497T>G	M1166R	Tumor	(Mosse et al., 2008)
22	c.3512T>A	I1171N	Tumor	(Mosse et al., 2008)
23	c.3520T>A	F1174I	Tumor	(Carén et al., 2008; Mosse et al., 2008)
23	c.3520T>G	F1174V	Tumor	(Chen et al., 2008; Janoueix-Lerosey et al., 2008)
23	c.3521T>G	F1174C	Tumor	(Chen et al., 2008; Janoueix-Lerosey et al., 2008)
23	c.3522C>A	F1174L	Tumor and cell lines	(Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008; Okubo et al., 2012)
24	c.3700G>A	A1234T	Tumor	(George et al., 2008)
24	c.3733T>G	F1245V	Tumor	(Bagci et al., 2012; Mosse et al., 2008)
24	c.3733T>A	F1245I	Tumor	(Carén et al., 2008)
24	c.3734T>G	F1245C	Tumor	(George et al., 2008; Mosse et al., 2008)
24	c.3735C>G	F1245L	Tumor	(Chen et al., 2008)
24	c.3735C>A	F1245L	Tumor	(Carén et al., 2008)
25	c.3749T>C	I1250T	Tumor	(Mosse et al., 2008)
25	c.3824G>A	R1275Q	Tumor and cell lines	(Bagci et al., 2012; Carén et al., 2008; Chen et al., 2008; De Brouwer et al., 2010; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008; Okubo et al., 2012)
25	c.3824G>T	R1275L	Tumor	(Janoueix-Lerosey et al., 2008)
25	c.3833A>C	Y1278S	Tumor	(De Brouwer et al., 2010; Janoueix-Lerosey et al., 2008)

3.4. Germline *ALK* mutations in hereditary NB

Hereditary NB is an autosomal dominant disease, with incomplete penetrance. This was the first example of a pediatric cancer associated with germline mutations in an oncogene (Mosse et al., 2008). *ALK* germline mutations are identified in approximately 50% of NB families (Table I.5) (Mosse et al., 2008). The mutations R1275Q (Janoueix-Lerosey et al., 2008; Mosse et al., 2008), R1192P (Bourdeaut et al., 2012; Janoueix-Lerosey et al., 2008; Mosse et al., 2008) G1128A (Mosse et al., 2008) and T1151R (Bourdeaut et al., 2012) have been described in families with NB.

The disease diagnosis at early age, presence of multifocal disease and familial recurrence of neuroblastic tumors are characteristics highly suggestive of a hereditary NB (Bourdeaut et al., 2012; Mosse et al., 2008). Mosse et. al. only found *ALK* mutations in NB families characterized by at least two affected individuals, related in first-degree. Bourdeaut et al. (2012) identified *ALK* germline mutations in 3 of 16 (18.75%) multifocal cases, suggesting that this clinical feature might be strongly indicative of an *ALK*-related predisposition in absence of familial history. However, younger age is not a major relevant

criterion indicative of *ALK*-related predisposition, as diagnoses have been reported at the age of three, 12 and 15 years in individuals with germline R1275Q and R1192P mutations (Janoueix-Lerosey et al., 2008).

The R1275Q *ALK* gene mutation is the most frequent mutation in hereditary NB, and is also one of the mutational hotspots in sporadic NB (Azarova et al., 2011; Mosse et al., 2008). This mutation was described as arising *de novo* in two families inherited either from an affected or unaffected parent (Mosse et al., 2008). On the other hand, the mutation R1192P previously described in four families was always inherited from an unaffected parent (Bourdeaut et al., 2012; Hallberg and Palmer, 2013; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). A *de novo* germline *ALK* mutation at position F1174, was found in a case of congenital NB with severe encephalopathy (de Pontual et al., 2011), indicating that it might cause embryonic lethality in most of the cases (Azarova et al., 2011; Bourdeaut et al., 2012; De Brouwer et al., 2010).

Table I.5: *ALK* germline mutations described in hereditary NB.

Exon	DNA	Protein	References
20	C.3260C>T	T1087I	(Chen et al., 2008)
21	c.3383G>C	G1128A	(Mosse et al., 2008)
22	c.3452C>T	T1151M	(Bourdeaut et al., 2012; George et al., 2008)
23	c.3575G>C	R1192P	(Bourdeaut et al., 2012; Janoueix-Lerosey et al., 2008; Mosse et al., 2008)
25	c.3824G>A	R1275Q	(Bourdeaut et al., 2012; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008)

3.5. ALK inhibitors in neuroblastoma treatment

The first ALK inhibitor tested in a clinical trial was Crizotinib, an oral bioavailable small molecule inhibitor approved for *EML4-ALK* positive NSCLC patients. It is an ATP-competitive molecule that prevents subsequent autophosphorylation necessary for further signal transduction. This drug was studied for the treatment of NB patients with either amplification or mutation in the *ALK* gene (Azarova et al., 2011). Some *in vitro* and *in vivo* studies have shown that Crizotinib is more potent in NB cell lines and xenografts with *ALK* amplification or the R1275Q mutation than in those with F1174L mutation, suggesting that the latter may promote resistance to the drug (Bresler et al., 2011). In a pediatric phase 1 and phase 2 trial of Crizotinib, two NB patients with *ALK* germline mutations responded to this therapy, but further studies are required (Mosse et al., 2013). However, more recent results suggest that the combination of Crizotinib with conventional chemotherapy may provide superior anti-tumor activity than each approach separately (Krytska et al., 2016).

Another ALK inhibitor is Entrectinib, a potent inhibitor of cell proliferation that induces cell death, causing cell cycle arrest. It was able of decrease growth and proliferation of *ALK*-amplified cell lines, however, shows low efficiency in *ALK* mutated cells (Aveic et al., 2016). Other ALK inhibitors have been studied, such as the potent second-generation *ALK* inhibitor Alectinib, which is active against some *ALK* mutations including, F1174L and R1275Q, indicating that this drug is able to overcome Crizotinib-induced chemoresistance (Lu et al., 2017).

More recently, Infarinato et al. (2016) described a novel ALK inhibitor with a superior potency than the others described so far. The ALK/ROS1 inhibitor PF-06463922 seems to exert activity in ALK-driven NB models with primary Crizotinib resistance. This new drug appears to be a key to overcome the ALK-driven therapy resistance, which is the biggest challenge in NB treatment with ALK inhibitors.

II. AIMS OF THE STUDY

II. AIMS OF THE STUDY

Due to the recently clinical implications of *ALK* changes in NB patients, the general aim of this work was to test for these alterations in NB patients diagnosed at the Portuguese Institute of Oncology of Porto (IPO-Porto) from 2003 to 2016. The specific aims were:

- To search for *ALK* fusion genes in neuroblastomas;
- To compare the pattern of amplification of the *ALK* and *MYCN* genes in neuroblastomas;
- To identify somatic and germline *ALK* tyrosine kinase domain (TKD) point mutations;
- To relate the *ALK* alterations with clinical outcome in neuroblastoma patients.

III. MATERIAL AND METHODS

III. MATERIAL AND METHODS

1. Patients and sample collection

A consecutive series of primary NB tumors from 46 patients treated at IPO-Porto from 2003 to 2016 and tested for *MYCN* amplification at the Department of Genetics was retrospectively analyzed. The clinical, genetical and pathological data of NB patients were recorded and are summarized in Table III.1.

Forty-one cell suspension samples of primary NB tumors were analyzed for *ALK* gene rearrangements by fluorescence in situ hybridization (FISH). Twenty-nine frozen fresh tumor samples were subsequently analyzed by Sanger sequencing for exons 20 to 28 of the *ALK* gene. Twenty-four patients were analyzed by both FISH and Sanger sequencing. Whenever possible, normal tissue was analyzed from patients presenting mutation in tumor samples.

Table III.1: Clinical and standard genetic information on the NB patients.

Sample Number	Age at diagnosis	Histology Classification	Cytogenetic results				Fixed cell suspension (FISH)	Frozen tissue (Sanger sequencing)
			1p	MYCN	11q	17q		
#1	1Y	Neuroblastoma	N	P	nd	nd	+	+
#2	2Y	Neuroblastoma	N	G	nd	nd	+	NO
#3	14Y	Ganglioneuroblastoma	N	P	nd	nd	+	+
#4	3Y	Neuroblastoma	L	N	nd	nd	+	NO
#5	1Y	Ganglioneuroblastoma	L	P	nd	nd	+	+
#6	10M	Ganglioneuroblastoma	N	L	nd	nd	+	+
#7	15Y	Ganglioneuroblastoma	L	P	nd	nd	NO	+
#8	14Y	Neuroblastoma	N	A	nd	nd	+	+
#9	14Y	Ganglioneuroblastoma	N	N	nd	nd	+	+
#10	1Y	Neuroblastoma	N	G	nd	nd	NO	+
#11	1Y	Ganglioneuroblastoma	N	P	nd	nd	+	+
#12	5M	Neuroblastoma	N	L	nd	nd	+	NO
#13	3Y	Neuroblastoma	L	G	nd	nd	+	NO
#14	3Y	Neuroblastoma	N	A	nd	nd	+	+
#15	9M	Neuroblastoma	N	P	nd	nd	+	NO
#16	0M	Neuroblastoma	N	G	nd	nd	+	+

#17	1M	Neuroblastoma	N	P	nd	nd	+	NO
#18	3Y	Neuroblastoma	L	A	nd	nd	+	NO
#19	1Y	Neuroblastoma	N	L	nd	nd	+	NO
#20	2Y	Neuroblastoma	N	P	L	G	+	+
#21	1Y	Neuroblastoma	nd	A	nd	nd	+	+
#22	4Y	Ganglioneuroblastoma	N	P	nd	nd	+	+
#23	2Y	Ganglioneuroblastoma	N	N	nd	nd	+	+
#24	6M	Neuroblastoma	L	P	N	N	+	+
#25	10M	Neuroblastoma	N	P	N	N	+	NO
#26	8Y	Neuroblastoma	N	N	nd	nd	+	+
#27	10M	Neuroblastoma	N	P	N	N	+	+
#28	3M	Neuroblastoma	L	A	N	N	+	+
#29	3Y	Neuroblastoma	L	A	nd	nd	+	NO
#30	7Y	Neuroblastoma	L	A	L	nd	NO	+
#31	3M	Neuroblastoma	N	P	N	N	+	+
#32	1M	Neuroblastoma	N	L	N	N	+	+
#33	7M	Neuroblastoma	N	N	N	N	+	+
#34	7Y	Neuroblastoma	N	N	N	N	+	+
#35	3Y	Neuroblastoma	L	G	nd	nd	+	NO
#36	3Y	Neuroblastoma	N	N	nd	nd	+	NO
#37	0M	Neuroblastoma	N	G	N	N	+	+
#38	2Y	Neuroblastoma	nd	P	nd	nd	+	NO
#39	2Y	Neuroblastoma	L	G	N	N	+	NO
#40	1Y	Neuroblastoma	L	A	L	N	+	NO
#41	5Y	Ganglioneuroblastoma	N	N	N	N	+	NO
#42	9M	Neuroblastoma	N	P	N	N	+	NO
#43	1Y	Ganglioneuroblastoma	L	A	N	N	+	+
#44	9M	Neuroblastoma	N	P	N	N	NO	+
#45	1Y	Neuroblastoma	L	G	N	G	+	+
#46	1Y	Neuroblastoma	nd	A	nd	nd	NO	+

M-months; Y-year; N-normal; L-loss; G-gain; P-polysomy; A-amplified; nd- not determined.

2. Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was performed with a dual color, break apart probe flanking the *ALK* gene, *ALK* (2p23) Break FISH probe, [Kreatech, Leica Biosystems, Nussloch, Germany] (Figure III.1). Slides were prepared fresh from cultured cells fixed with methanol:acetic acid. Co-denaturation was performed at 80°C for 8 min, followed by hybridization at 37°C in humidified chamber (ThermoBrite denaturation/hybridization system [Leica Biosystems, Nussloch, Germany]) for 18 hours. Post-hybridization washes were done using 2xSSC/0.1% IGEPAL [Sigma Aldrich] solution at room temperature (RT) for 30 minutes, followed by 0.4 x SSC/ 0.3% IGEPAL at 74 °C for 2 min and 2 x SSC/ 0.1% IGEPAL for 5 minutes, at RT. Slides were dehydrated by passages through an increasing series of alcohols, 70%, 85% and 100%, 2 minutes each and air dried. DAPI (4', 6-diamidino-2-phenylindole) [Vector Laboratories, Burlingame, California, USA] was applied as a counterstain and results were evaluated with a GSL-120 Automated Cytogenetics Platform with a CytoVision Software Version number 7.4 [Leica Biosystems]. For each sample, at least 50 intact non-overlapping nuclei were scored.

Fluorescence images corresponding to DAPI, Spectrum Green and Spectrum Orange were automatically captured and relative copy number alteration in 2p23 region was assessed as previously described (Subramaniam et al., 2009).

Since centromeric chromosome 2 copy number was available from previous *MYCN* analysis, the status of *ALK* was calculated for each sample, according to *MYCN* status criteria (Ambros et al., 2009). An abnormal signal pattern was considered representative when present in a minimum of 10% of tumor cells.

Cases were categorized as:

- *ALK* gain (G), when the number of *ALK* gene signals was one- to four-times greater than the CEP2 signals;
- *ALK*-amplified (A), when the number of *ALK* gene signals was four-times greater than the CEP2 signals;
- *ALK* loss/imbalance (L), when the number of *ALK* gene signals was less than that of CEP2 signals;
- Chromosome 2 polysomy (P), when both CEP2 and *ALK* signals were increased.

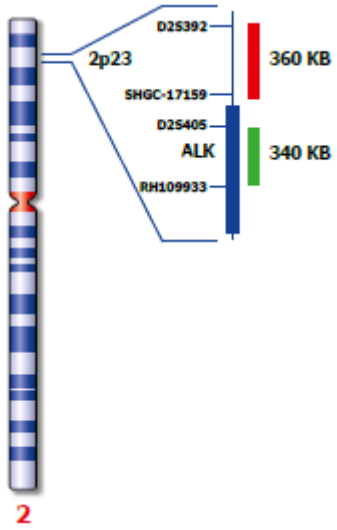

Figure III.1: *ALK* (2p23) break FISH probe to detect rearrangements involving the *ALK* gene. Red critical region represents the distal *ALK* gene region direct-labeled with PlatinumBrigh^{TM}550 (3′ALK SpectrumOrange) and green critical region represents the proximal *ALK* gene region direct-labeled with PlatinumBrigh^{TM}495 (5′ALK SpectrumGreen).

3. DNA extraction and quantification

3.1. Extraction of genomic DNA from frozen fresh samples

A piece of frozen tumor was minced with a scalpel and transferred to a 50ml falcon. Four ml of SE buffer (75mM NaCl; 25 mM EDTA), 400µl of SDS (Sodium Dodecyl Sulfate) 10% and 50µl of proteinase K [Gibco Invitrogen, Carlsbad, CA, USA] (10ng/µl) were added, followed by incubation at 55ºC overnight. One ml of NaCl (6M) was added to the lysed sample and incubated at 55ºC for 10 minutes. An equal volume of chloroform was added, gently mixed for 30 minutes and centrifuged at 4000 rpm for 10 minutes. The aqueous DNA solution was transferred to a 15ml falcon and an equal volume of isopropanol 100% was added. The precipitated DNA was transferred to an eppendorf with 70% ethanol. Subsequently, ethanol was discarded and air dried. Finally, the DNA was eluted in ddH_2O and was quantified by spectrophotometry with NanoDrop ND-1000® [NanoDrop Technologies, Wilmington, DE, USA].

3.2. Extraction of genomic DNA from paraffin-embedded samples

Tumor areas containing normal cells were delimited by a pathologist in the hematoxylin and eosin (H&E) stained slides of each sample. The corresponding unstained slides were immersed in xylene [Sigma-Aldrich, Steinheim, Germany] and then in ethanol 100% [Merck, Darmstadt, Germany] for 5 minutes each to remove the paraffin. Tumor areas, which were previously delimited by comparison with the correspondent H&E stained slides, were macro dissected and transferred to a centrifuge tube and the extraction was performed with a

cobas® DNA Sample Preparation Kit, used for manual specimen preparation of formalin-fixed paraffin-embedded tumor (FFPE) tissues [Roche Diagnostics, Mannheim, Germany], according to standard procedures. Finally, the DNA was quantified by spectrophotometry with NanoDrop ND-1000® [NanoDrop Technologies, Wilmington, DE, USA].

4. DNA sequencing

Mutation screening in the tyrosine kinase domain of *ALK* (exons 20 to 28) was performed by Sanger sequencing. For this purpose, DNA was amplified in a solution containing 1x PCR gold buffer [Thermo Fisher Scientific, Foster city, CA, USA] (150 mM Tris-HCl, 500 mM KCl), 1.5 mM of MgCl2 [Thermo Fisher Scientific], 0.5 mM dNTP mix [Thermo Fisher Scientific], 0.17 mM of each primer (reverse and forward) [frilabo, Portugal], 1 U of Taq DNA polymerase [Thermo Fisher Scientific], in a final reaction volume of 20 µL. The primer sequences were used from Chen et al. (2008) and are summarized in Table III.2. PCR reaction was performed in a thermocycler [Gene Amp PCR Systern 9700, Perkin-Elmer, Waltham, Massachusetts, USA] according to the following conditions: an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 95°C for 45 seconds, annealing step at 58°C for 45 seconds and a 45 seconds extension step at 72°C. A final extension step was done at 72°C for 10 min. Amplified PCR products were then analyzed by electrophoresis in a 2% (w/v) agarose gel [Gibco Invitrogen] stained with green safe [Sigma-Aldrich] 0.05 µL/mL.

Table III.2: Set of primers used to amplifying exons 20 to 28 (Chen et al., 2008).

Exon	Direction	Primers pairs
20	F	AGGAAGTGGCCTGTGTAGTG
	R	ATAACATTCAGCCCCTACAC
21-22	F	TGACTCTGTCTCCTCTTGTC
	R	TTAGAAACCTCTCCAGGTTC
23	F	AAGATTTGCCCAGACTCAGC
	R	TGTCCTTGGCACAACAACTG
24	F	AGATTTCCCTCCTCTCACTG
	R	ATGTGAGCCCTTGAGATCTG
25	F	TAGTGATGGCCGTTGTACAC
	R	CCAGGAGATGATGTAAGGGA
26	F	GGCAGATGCTTAATGCCATC
	R	CTCCCGGCTTAGAGTATAGA
27	F	TGGGTGTGTCTATATCCATC
	R	GTAACTAGCAGAAGTGTTCC
28	F	CCCTCAACGTATTCGTTGCA
	R	ACTCTGACTGGCTTGACCTA

F-Forward; R-Reverse

Subsequently, the PCR products were purified using the ExoSAP-IT method for the removal of primers and dNTPs in excess. Samples were purified adding 2 µL of ExoSAP solution, which consists in Exonuclease I [Thermo Fisher Scientific] (20 U/µL) and Fast Thermosensitive Alkaline Phosphatase [Thermo Fisher Scientific] (1 U/µL), in a proportion of 1:2, to 5 µL of the PCR product, followed by incubation at 37ºC for 50 minutes, and enzyme inactivation at 80ºC for 15 minutes.

The purification was followed by the sequencing reaction, in which the BigDye® Terminator v1.1 or v3.1 Cycle Sequencing Kit [Applied Biosystems, Foster City, CA, USA] was used. The reaction consisted on mixing 3.4 µL of sequencing buffer, 0.5 µL of Big Dye® Terminator v1.1 or v3.1, containing dNTPs, ddNTPs-fluorocromes, MgCl2 and Tris-HCl buffer, 0.32 µL of one of the primers (forward or reverse), and bidestilled sterile water [B. Braun, Foster City, CA, USA] and 1 µL of the previously purified DNA to reach a final reaction volume of 10 µL. The sequencing reaction was performed and consisted of an initial denaturation step at 95ºC for 4 minutes, followed by 35 cycles of denaturation at 96ºC for 10 seconds, annealing at 50ºC for 10 seconds and extension at 60ºC for 2 minutes, with a final extension of 60ºC for 10 minutes. In order to remove excess of dNTPs, labeled ddNTPs, and non-incorporated primers, the sequencing products were purified with Illustra Sephadex® G-50 fine [GE

Healthcare, Life Sciences, Cleveland, USA], according to standard procedures. After purification, 15 µL of Hi-Di™ Formamide [Applied Biosystems] were added to the sequencing products to help stabilize the single stranded DNA. The products were then analyzed in a 3500 Genetic Analyzer [Applied Biosystems] by capillary electrophoresis. The electropherograms of each sample were analyzed with the Sequencing Analysis Software v5.4 [Applied Biosystems]. All of them were examined at least twice, reviewed manually and with the Mutation Surveyor® DNA Variant Analysis Software v4.0.8 [Softgenetics, State College, PA, USA].

A second PCR amplification was performed in all positive samples, followed by DNA Sanger sequencing of both strands. All *ALK* variants were described according to the LRG_488 (NM_004304.3) and to the Human Genome Variations Society guidelines.

IV. RESULTS

IV. RESULTS

1. Assessment of *ALK* rearrangements in NB patients

ALK rearrangements were screened with a dual-color break-apart probe FISH in 41 tumor samples from patients with NB. The results are summarized in Table IV.1 and illustrated in Figure IV.1.

Table IV.1: *ALK* alterations assessed by FISH in NB patients.

Sample Number	ALK signal	CEP 2 signal*	ALK alterations
#1	3~4 F (81%)	3~4	polysomy
#2	5~6 F (90%)	3~4	gain
#3	3 F (99%)	3	polysomy
#4	3~4 F (18%)	2	gain
#5	3~4 F (70%)	3~4	polysomy
#6	3~4 F (81%)	4~5	loss/imbalance
#8	3~4 F (16%)	3~4	polysomy
#9	2 F (100%)	2	normal
#11	3~4 F (100%)	3~4	polysomy
#12	3 F (100%)	3~4	loss/imbalance
#13	4~6 F (94%)	3~6	gain
#14	3~5 F (41%)	3~4	gain
#15	3~4 F (80%)	3~4	polysomy
#16	3~5 F (100%)	3~4	gain
#17	3 F (100%)	3	polysomy
#18	3~5 F (100%)	3~5	polysomy
#19	3~6 F (87%)	4~8	loss/imbalance
#20	3~4 F (20%)	3~10	loss/imbalance
#21	3~5 F (66%)	3~4	gain
#22	3 F (100%)	3	polysomy

#23	2 F (100%)	2	normal
#24	3~4 F (100%)	3~4	polysomy
#25	3~4 F (100%)	3~4	polysomy
#26	2 F (100%)	2	normal
#27	3~4 F (100%)	3~4	polysomy
#28	3 F (34%)	2	gain
#29	3~6 F (nd)	2~5	gain
#31	3 F (100%)	3	polysomy
#32	3~4 F (79%)	4~5	loss/imbalance
#33	2 F (93%)	2	normal
#34	2 F (91%)	2	normal
#35	3~4 F (100%)	2	gain
#36	2 F (92%)	2	normal
#37	3~4 F (100%)	3	gain
#38	3 F (100%)	3	polysomy
#39	3~4 F (15%)	3	gain
#40	3~4 F (11%)	2	gain
#41	2 F (100%)	2	normal
#42	3 F (96%)	3	polysomy
#43	Amp (81%)	2	amplified
#45	6~8 F (100%)	3~4	gain

*These data was available from previous analyses.
F-fusion

No translocations or inversions were found in our cohort, however, aberrant copy number of the *ALK* gene was observed in 19 cases (46.3%), including *ALK* amplification in one of 41 (2.4%), *ALK* gain in 13 of 41 (31.7%) and *ALK* loss/imbalance in five of 41 (12.2%). Chromosome 2 polysomy was observed in 15 cases (36.6%) and normal signal pattern was observed in the remaining seven cases (17.1%).

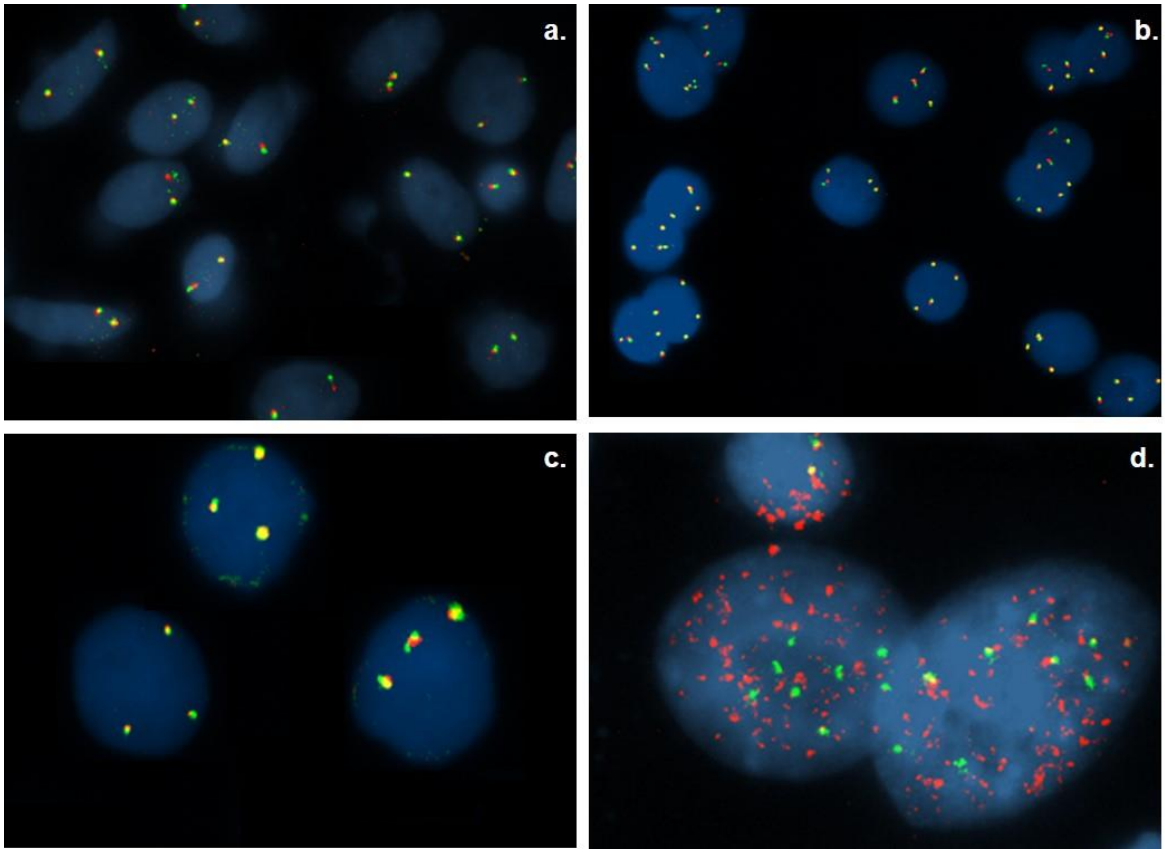

Figure IV.1: Representative FISH images from four selected NB tumors using a dual color, break apart probe targeting 3′ALK and 5′ALK, labeled orange and green, respectively. a. normal nuclei, presenting two *ALK* gene signals; **b.** nuclei presenting three to four *ALK* gene signals; **c.** nuclei presenting three *ALK* gene signals; **d.** nuclei with amplification of 3'ALK region.

1.1. Clinicopathological characteristics of patient with *ALK* amplification

The patient with *ALK* amplification (81% of the cells) was a female child aged 14 months old diagnosed with a neuroblastoma (stroma-poor, undifferentiated, low MKI and unfavorable histology), located in the right adrenal medulla, with invasion of adjacent tissues, regional lymph nodes and the bone marrow. The previous cytogenetic analysis showed *MYCN* amplification in 78% of cells, similar to the *ALK* pattern found in this study, as well as deletion of 1p36 region in 54% of cells. According to INRG risk stratification, the tumor was classified as a high-risk neuroblastoma. After neoadjuvant chemotherapy and surgery, maturation of tumor cells induced by chemotherapy was observed, being the tumor reclassified as a ganglioneuroblastoma, subtype “intermixed” and favorable histology. The patient died 12 months after diagnosis.

2. *ALK* mutations in NB patients

DNA samples from 29 children diagnosed with NB were screened for somatic mutations in exons 20 to 28 of the *ALK* gene by Sanger sequencing. Four different mutations were found in five cases, corresponding to a frequency of 17.2% in this cohort. Two of these were considered germline mutations, given that they were detected in normal tissue (Table IV.2).

Table IV.2: Mutations found in the *ALK* gene.

Sample number	c.DNA designation	Protein designation	Exon number	Effect	Germline/ somatic
#1	c.3824G>A	p.(Arg1275Gln)	Exon 25	Missense	Somatic
#5	c.3522C>G	p.(Phe1174Leu)	Exon 23	Missense	Somatic
#26	c.3575G>C	p.(Arg1192Pro)	Exon 23	Missense	Germline
#33	c.3520T>A	p.(Phe1174Ile)	Exon 23	Missense	Somatic
#44	c.3824G>A	p.(Arg1275Gln)	Exon 25	Missense	Germline

The mutation found in patients #1 (somatic) and #44 (germline) consists of a nonsynonymous substitution of a Guanine for an Adenine (transition, c.3824G>A) at the second position of codon 1275 (CGA→CAA), resulting in a nonconservative substitution of an Arginine for a Glutamine, p.(Arg1275Gln) (R1275Q) (Figure IV.2a, b).

The mutation found in patient #5 (somatic) consists of a nonsynonymous substitution of a Cytosine for a Guanine (transversion, c.3522C>G) at the third position of codon 1174 (TTC→TTG), resulting in a nonconservative substitution of an Phenylalanine for a Leucine, p.(Phe1174Leu) (F1174L) (Figure IV.2c).

The mutation found in patient #26 (germline) consists of a nonsynonymous substitution of a Guanine for a Cytosine (transversion, c.3575G>C) at the second position of codon 1192 (CGG→CCG), resulting in a nonconservative substitution of an Arginine for a Proline, p.(Arg1192Pro) (R1192P) (Figure IV.2d).

The mutation found in patient #33 (somatic) consists of a nonsynonymous substitution of a Thymine for an Adenine (transversion, c.3520T>A) at the first position of codon 1174 (TTC→ATC), resulting in a nonconservative substitution of an Phenylalanine for an Isoleucine, p.(Phe1174Ile) (F1174I) (Figure IV.2e). This mutation has been described in sporadic tumors (Carén et al., 2008; Mosse et al., 2008).

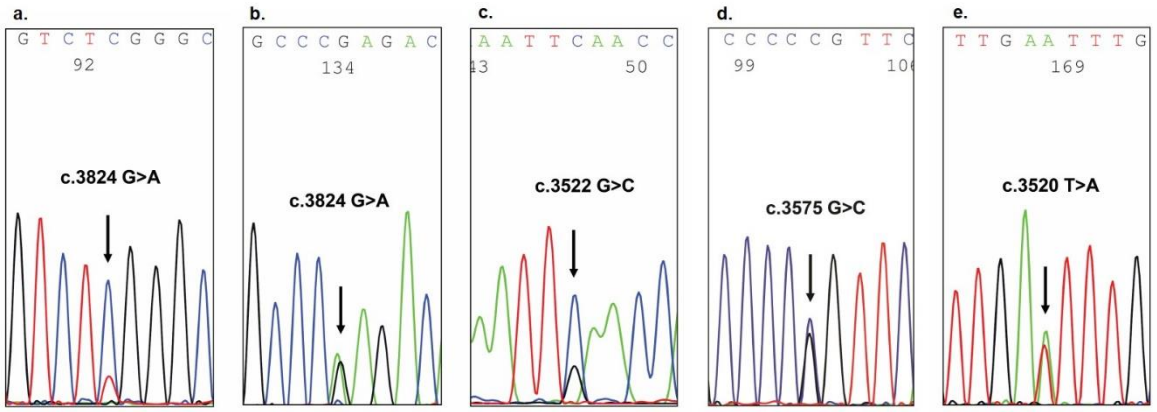

Figure IV.2: DNA sequence electropherograms obtained from NB tumor samples: a. patient #1: *ALK* c.3824 G>A (reverse strand), p.(Arg1275Gln) (R1275Q); **b.** patient #44: *ALK* c.3824 G>A (forward strand), p.(Arg1275Gln) (R1275Q) **c.** patient #5: c.3522C>G (forward strand), p.(Phe1174Leu) (F1174L); **d.** patient #26: c.3575G>C (forward strand), p.(Arg1192Pro) (R1192P); **e.** patient #33: c.3520T>A (reverse strand), p.(Phe1174Ile) (F1174I).

2.1. Clinicopathological characteristics of patients with *ALK* mutations

Patient #1, with the somatic *ALK* mutation c.3824G>A**,** p.(Arg1275Gln)**,** is a female child aged 21 months old diagnosed with a neuroblastoma (stroma-poor, poorly differentiated, intermediate MKI, favorable histology), located in the upper mediastinum. The tumor presented metastization at supraclavicular ganglia at the time of diagnosis. This tumor did not present *MYCN* amplification or 1p deletion and we did not find *ALK* cytogenetic alterations, however, there was a chromosome 2 polysomy. At the time of writing, the patient was under follow-up, with no evidence of disease.

Patient #5, with the somatic *ALK* mutation c.3522C>G**,** p.(Phe1174Leu)**,** is a male child aged 20 months old diagnosed with a thoracic ganglioneuroblastoma (stroma-dominant, favorable histology), with local invasion. The tumor presented deletion of 1p and chromosome 2 polysomy. At the time of writing, and after 13 years of follow-up, the patient is free of NB, however, he is under treatment for a cervical myxoid liposarcoma diagnosed at the age of 13.

Patient #26, with the germline *ALK* mutation c.3575G>C**,** p.(Arg1192Pro), is an eight years old female child, diagnosed with a retroperitoneal neuroblastoma (stroma-poor, undifferentiated, high MKI and unfavorable histology). The tumor did not present *MYCN* amplification, 1p deletion or *ALK* cytogenetic alterations. The patient died 28 months after diagnosis. There is no history of NB in this family.

Patient #33, with the somatic *ALK* mutation c.3520T>A, p.(Phe1174Ile), is a female child aged eight months old diagnosed with an abdominal neuroblastoma (stroma-poor, undifferentiated, high MKI and unfavorable histology). The tumor did not present *MYCN*

amplification, deletion of 1p and 11q, gain of 17q or *ALK* cytogenetic alterations. After neoadjuvant chemotherapy and surgery, which induced maturation of tumor cells, the tumor was reclassified as a neuroblastoma, stroma-poor, differentiating, with low MKI and favorable histology. After 20 months of follow-up, a relapse was detected, and classified after surgery as a ganglioneuroma, maturing, with low MKI and favorable histology.

Patient #44, with the germline *ALK* mutation c.3824G>A**,** p.(Arg1275Gln)**,** is a female child diagnosed at the age 9 months old with an abdominal neuroblastoma, presenting metastases in the femur and in the facial bones. The tumor did not present *MYCN* amplification, deletion of 1p or 11q, or gain of 17q. The FISH analysis of *ALK* was not performed in this case. At the present time, the patient is being prepared for an AHSCT. Family history is not known at the time of writing.

V. DISCUSSION

V. DISCUSSION

The discovery of a variety of genetic alterations in tumors leading to oncogenesis and the development of target-specific therapies have improving patients' outcomes in different malignancies. Rearrangements, mutations and amplification of the *ALK* gene have been described in a range of tumors, including ALCL, IMT and NSCLC (Hallberg and Palmer, 2013). This indicates the ALK protein as a powerful biological marker and a therapeutic target in malignancies in which *ALK* influences carcinogenesis (Bresler et al., 2011; Solomon et al., 2014). Relatively recent findings have suggested that the *ALK* gene may be activated by amplification or specific mutations targeting the tyrosine kinase domain (TKD) in NB patients, presumably contributing to tumor development (Bagci et al., 2012; Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Miyake et al., 2002; Mosse et al., 2008; Osajima-Hakomori et al., 2005; Subramaniam et al., 2009). Moreover, germline activating mutations in *ALK* gene are responsible for more than half of NB hereditary cases (Mosse et al., 2008). In the present study, we aimed to search for the *ALK* fusion genes, to compare the patterns of amplification of the *ALK* and *MYCN* genes, to identify somatic and germline *ALK* TKD point mutations, and to relate the *ALK* alterations with clinical outcome in NB patients.

Using the FISH methodology, we searched for *ALK* chromosome alterations in forty-one patients from all clinical stages. The *ALK* gene is involved in the initiation and progression of different malignancies, being often activated by translocations (Hallberg and Palmer, 2013). In the present study, we did not observe any *ALK* rearrangement, characterized by a split of the *ALK* probe, suggestive of a translocation or inversion with breakpoint within the *ALK* gene. These rearrangements, although uncommon, have been described in previous studies, although in larger cohorts and using more sensitive methodologies such as SNP arrays (Cazes et al., 2013; Fransson et al., 2015).

However, we found cytogenetic aberrations in 46.3% of the cases. Amplification was detected in one high-risk NB patient (2.4%), which is in accordance with previous studies that report frequencies of 1 to 3% in advanced stages, using FISH (Osajima-Hakomori et al., 2005; Subramaniam et al., 2009; Wang et al., 2013) or genome-wide scanning approaches (Bagci et al., 2012; Carén et al., 2008; Chen et al., 2008; De Brouwer et al., 2010; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). This case also presented *MYCN* amplification, which is in agreement with previous studies that suggested the synchronic co-amplification of the two genes (Bagci et al., 2012; De Brouwer et al., 2010; George et al., 2008; Subramaniam et al., 2009), which is compatible with the similar pattern of amplification of both genes in our patient. Although *ALK* amplification without *MYCN*

amplification is very rare, having only been detected in three cases (Chen et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008), Chen *et al.* (2008), showed that amplification of *ALK* and *MYCN* loci may occur in separated events.

The copy number gain without amplification was found in our cohort in 31.7% of the cases. The *ALK* copy number gain was described previously in 17 to 45% of the cases, being highly associated with more advanced clinical stages and with non-hyperdiploid tumors with a worse outcome (Bagci et al., 2012; Carén et al., 2008; Chen et al., 2008; Mosse et al., 2008). However, Wang et al. (2013) showed a better outcome of patients with *ALK* copy number gain, which may be related with the fact that the majority of these cases also had gain of centromere 2, a feature that suggests a polyploid karyotype that is known to confer a favorable prognosis in NB patients. In this cohort, we detected 36.6% of tumors with chromosome 2 polysomy, which is an indicator of a better outcome of patients without other adverse prognostic features.

Additionally, we found five cases (12.2%) presenting loss/imbalance of the *ALK* locus, characterized by increased centromere of chromosome 2 probe signals, and lower number of the *ALK* gene signals. This cytogenetic category was reported for the first time by Subramaniam et al. (2009). Four of five cases with *ALK* loss/imbalance also showed *MYCN* loss/imbalance. This can be explained by gain of a rearranged chromosome 2 with relative *ALK* and *MYCN* allelic loss (Subramaniam et al., 2009). Interestingly, neither deletion of 1p and 11q or duplication of 17q showed correlation with *ALK* copy number alterations, which is in agreement with the results showed by Subramaniam et al. (2009).

In this study, we searched for *ALK* mutations in twenty-nine NB tumors by Sanger sequencing and we found a frequency of 17.2%, which is higher when compared to previous studies that described frequencies of 7 to 14% (Azarova et al., 2011). Four of the five *ALK* mutations identified occurred at positions R1275 and F1174, which are two mutational hotspots in sporadic tumors. A meta-analysis of 709 NB tumors identified these mutations in tumors of all clinical stages and genomic profiles, in 49% and 34.7% of sporadic NB, respectively. However, they described a significantly higher frequency of F1174 mutations in *MYCN*-amplified tumors, which might explain the inferior survival reported by them in these patients. Additionally, Schonherr et al. (2012) proposed that *MYCN* may cooperate with *ALK* activating mutations to increase cell transformation and synergistically contribute to tumor development, since the *ALK* gene is a direct transcriptional target of *MYCN* and a downregulation or inhibition of *ALK* results in a decreased *MYCN* expression (Hasan et al., 2013; Schonherr et al., 2012). In the present study, we did not find any *ALK* mutations in any of *MYCN* amplified cases, which may be due the small number of cases analyzed.

The mutation R1275Q found in two of five cases was previously described in several studies as one of the most frequent mutations occurring in both sporadic (Carén et al., 2008;

Chen et al., 2008; De Brouwer et al., 2010; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008) and hereditary NB cases (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). This mutation has been associated with higher autophosphorylation and phosphorylation of downstream targets, such as ERK1/2 and Akt with high capacity of transformation in NIH 3T3 and Ba/F3 assays (Chen et al., 2008; De Brouwer et al., 2010; George et al., 2008). Studies performed by Ueda et al. (2016), demonstrated that the mutation R1275Q alone was not sufficient to tumor development in mice, but NB development occurred when the mutation was concurrent with MYCN expression. They suggested that this activating mutation contribute to the pathogenicity of NB by downregulation of the expression of extracellular matrix (ECM)- and basement membrane (BM)- associated genes, enhancing the ability of tumor MYCN-expressing cells to migrate and invade. Furthermore, the *ALK*-inhibitor agent-crizotinib showed almost completely suppression of *ALK* R1275Q mutated tumors growth in xenograft models (Bresler et al., 2011; Ueda et al., 2016).

The mutation F1174L found in one of five patients has been almost exclusively detected in sporadic tumors (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008; Okubo et al., 2012), probably reflecting the higher kinase activity and cell transforming capacity of this mutation. The F1174L mutation shows a high degree of autophosphorylation and phosphorylation of downstream targets involved in PI3K/Akt/mTOR and MAPK signal transduction pathways, and is preferentially associated with *MYCN* amplified cases (Berry et al., 2012; Chen et al., 2008; De Brouwer et al., 2010; George et al., 2008). Berry et al. (2012) demonstrated that this mutation did not induce tumor development by itself, however, it potentiates the oncogenic effects of *MYCN*, contributing to its stabilization and, consequently, to the development of more aggressive tumors. Studies in cell lines have showed that this mutation is extremely sensitive to the potent ALK inhibitor TAE684 (George et al., 2008) whereas it seems to be predictive of resistance to crizotinib (Bresler et al., 2011). Berry and colleagues associated this resistance with the incomplete inhibition of the key signaling cascades induced by F1174L mutation. Treatment with crizotinib leads to reduction of pALK and pAkt levels, however, it lacks any effect in pERK or activated mTOR signaling. Therefore, patients carrying the F1174L mutation may benefit from a combined therapy with crizotinib and torin2, an ATP-competitive inhibitor of mTOR (Berry et al., 2012).

The mutation F1174I found in one of five tumors is located at the same position as the F1174L mutation, however, it has been described less frequently (Berry et al., 2012; Carén et al., 2008; Mosse et al., 2008). Similarly to *ALK* F1174L, this mutation is expected to be pathogenic, however, no functional studies have been performed for this specific mutation.

In this study, we identified two germline mutations, the R1275Q and the R1192P initially identified in the tumor and subsequently confirmed to be present also in the germline. The germline R1275Q mutation is the most frequent mutation described in hereditary NB (Bourdeaut et al., 2012; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). Mosse et. al. (2008) who studied 20 NB families, found the R1275Q *ALK* mutation in five of eight families with *ALK* germline mutations. Furthermore, all families with this mutation showed at least two affected first-degree individuals, although several asymptomatic carriers were also identified, suggesting incomplete penetrance (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). The patient with the R1275Q germline mutation detected in the present work was diagnosed at the age of nine months old, with metastatic disease, which is suggestive of hereditary NB.

The germline R1192P mutation found in one case has only been described in hereditary NB cases, being the second commonest germline mutation detected in NB patients. The mutation was previously detected in two of the eight families found with *ALK* germline mutations by Mosse and colleagues (Mosse et al., 2008). Interestingly, this mutation was always inherited from an unaffected parent, and the age at diagnosis ranged from three months to 12 years (Bourdeaut et al., 2012; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). The patient carrying the R1192P germline mutation in the present cohort was diagnosed at the age of eight years old, with an unfavorable tumor. Additionally, to our knowledge there is no history of NB in this family. However, given the possibility of incomplete penetrance, we cannot exclude the existence of unaffected carriers in this family. Moreover, cases have been described of unaffected carriers that had spontaneously regressive tumors or asymptomatic stroma-rich tumors (Bourdeaut et al., 2012).

Concluding, *ALK* alterations are a frequent event in NB patients, either by cytogenetic events or point mutations, being a potential clinically useful predictive and prognostic biomarker that may be a therapeutic target in a subset of patients. The results obtained in this work suggest the need of new molecular diagnostic recommendations for NB patients, which will allow the clinical evaluation of *ALK* inhibitors. However, further studies are needed to identify which patients are more likely to respond to ALK inhibitors. Additionally, we also found two children with germline *ALK* mutations, which can help direct screening of healthy relatives with NB predisposition and of second primary in the patients, although further studies are necessary to establish the penetrance of the various germline mutations.

VI. FUTURE PERSPECTIVES

VI. FUTURE PERSPECTIVES

This study may benefit from further analysis to support our conclusions and to allow a more complete characterization of the *ALK* gene in NB patients. Thus, we plan:

- To increase our cohort to 56 NB patients;
- To analyze by FISH and Sanger sequencing formalin fixed paraffin embedded tissue from the remaining cases;
- To evaluate the ALK expression pattern by immunohistochemistry (IHC) in all cases, to compare the protein expression between *ALK* altered and normal cases.

VII. BIBLIOGRAPHY

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SUPPLEMENTARY TABLES

Supplementary Table 1: The International Neuroblastoma Pathology Classification (Shimada system) [adapted from (Shimada et al., 1999)].

Schwannian development	Degree of neuroblast differentiation	MKI	Age	Histology
Neuroblastoma stroma-poor	Undifferentiated	Low	All ages	unfavorable
		Intermediate		
		High		
	Poorly differentiated	Low	< 18 months	favorable
		Intermediate	< 18 months	favorable
			> 18 months	unfavorable
		High	All ages	
	Differentiating	Low	< 5 years	favorable
			> 5 years	unfavorable
		Intermediate	< 18 months	favorable
			> 18 months	unfavorable
		High	All ages	
Ganglioneuroblastoma, intermixed stroma-rich				favorable
Ganglioneuroma, Stroma-dominant	Maturing			favorable
	Mature			
Ganglioneuroblastoma, nodular Stroma-rich/ stroma-dominant and stroma-poor				unfavorable

Supplementary Table 2: International Neuroblastoma staging system (Brodeur, et al. 1993).

Stage	Definition
1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive).
2A	Localized tumor with complete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically.
2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
3	Unresectable unilateral tumor infiltrating across the midline with or without regional lymph nodes involvement; or localized unilateral tumor with contralateral regional lymph nodes involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph nodes involvement.
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver skin and or other organs (except as defined for stage 4S).
4S	Localized primary tumor (as defined for stage 1, 2A or 2B), with dissemination limited to skin, liver and/or none marrow (<10% tumor involvement). Limited to infants < 1 year of age).